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We have evaluated two strategies for identifying inhibitors of the DNA repair pathway. We used the yeast reverse two hybrid system to screen for inhibitory peptides of the Rad51 protein in order to disrupt the homologous recombination pathway from a plasmid library coding for 15 amino acid peptides. While we succeeded in generating a library of sufficient complexity, because of inherent technical issues involved we were not successful in identifying the inhibitory peptides.

For identifying inhibitors of the Ku70/80 complex (the important component of the NHEJ pathway) we used structure-based virtual ligand screening using DOCK. We identified four compounds from the in-silico screen as also having biochemical activity in a Ku70/80 DNA binding assay. Two of the compounds effectively sensitized cancer cells to DNA damaging agents while having little toxicity themselves. Our results demonstrate the validity and importance of both our target (DNA repair) as well as the approach we used to identify inhibitors of the target (DOCK). Targeting the DNA repair pathway is effective in sensitizing cells to DNA damaging agents such as topoisomerase inhibitors and ionizing radiation. Structure-based drug screening using virtual libraries may be a useful and economical way to identify inhibitors of large multi subunit complexes.

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INTRODUCTION

Despite considerable advances in our understanding of the causes and molecular basis of cancer, it is still a major cause of death in western nations. In 2004 alone there will be more than 1.3 million new incidences of cancer and more than half a million Americans will succumb to these diseases (American Cancer Society 2004 Statistics, http://www.cancer.org/downloads/STT/CAFF_finalPWSecured.pdf). The 5-year mean survival rate is less than 65%. Next to heart disease, cancer is also the second leading cause of death. Treatment of cancer usually involves sugical excision of the tumor (in case of some solid tumors like breast cancer) followed by a combination of radiation therapy and chemotherapy. Radiation therapy is mainly used to treat localized solid tumors, such as cancers of the skin, tongue, larynx, brain, breast, or uterine cervix and involves targetting the tumor area with ionizing radiation. Tumor cell killing occurs through extensive DNA damage. Chemotherapy for cancer involves an extensive number of targets ranging from non-specific DNA damaging drugs (alkylating agents, crosslinking agents like cisplatin, double strand DNA break-inducing agents like topoisomerase inhibitors), drugs acting on cell division (like taxol), to drugs acting on cancer specifc targets like Gleevec® (cAbl kinase inhibitor), Iressa® (anti-angiogenic agent, epidermal growth factor receptor tyrosine kinase EGFR-TK inhibitor) and estrogen receptor antagonists like tamoxifen.

Summary of Objectives

This project was an attempt to apply novel approaches in drug screening to identify new leads for investigation. We utilized genomics screening and existing software tools in chem-informatics to quickly evaluate, screen and identify inhibitors of the DNA repair pathway. To identify inhibitors of the homologous recombination pathway, we used the reverse yeast two-hybrid screen and a plasmid library coding for random 15 amino acid peptides to screen for inhibitory peptides of RAD51 self association. To inhibit the non-homologous end-joining pathway (NHEJ), we targeted the DNA binding protein complex Ku70/80. We used the freely available virtual ligand screening software DOCK (ver 4.0) to screen two databases of small molecule compound structures for this purpose. The highest scoring hits from this analysis were then tested in in-vitro biochemical assays for activity. The leads with inhibitory activity were then evaluated in cell culture for validation.

BODY

A. SCREENING FOR INHIBITORY PEPTIDES OF RAD51 SELF-ASSOCIATION

Tumorigenesis is the result of multiple genetic changes. Although cells are subject to a multitude of environmental and chemical factors, their robust repair machinery is able to repair most of the damage and, if not, at least program the cell to undergo apoptosis, thereby preventing their uncontrolled proliferation with damaged DNA. Most therapeutic agents for breast cancer also function by causing DNA damage, either directly (ionizing radiation) or indirectly (topoisomerase inhibitors). The efficacy of these drugs are directly dependant on the cell's ability to repair the damaged DNA quickly and efficiently. Therefore any agent that can specifically target the tumor's repair machinery can be used to sensitize it to the DNA damaging agent.

Both ionizing radiation and topoisomerase inhibitors cause double strand breaks in the DNA. These double strand breaks (DSBs) are repaired by one of two major pathways- homologous recombination (HR) pathway and the nonhomologous end joining (NHEJ) pathway. The efficiency of cell killing by the DSB causing agents is dependant on the efficiency of DNA repair mechanisms in the tumor cell. Cells defiecient in one or more of the DNA repair mechanisms are sensitive to agents that cause DNA damage(1, 2). Thus these DNA repair pathways are attractive targets for inhibitor screening for use as sensitizing agents to DNA damaging drugs. We decided to use two distinct approaches for targeting these pathways. We initially used a reverse two hybrid screen to screen a random library coding for 15 a.a peptides to screen for inhibitory peptides for RAD51 self association. We then used a virtual ligand screening approach targeting the DNA binding pocket of the Ku70/80 dimer to identify potential inhibitors of the NHEJ pathway.

Homologous recombination is one of the important DNA double strand break repair pathways. While its prominent function is the exchange of information during meiosis, its has been shown to be a key pathway in DNA repair in bacteria and yeast(3). In bacteria, the Rec A protein binds single-stranded DNA and drives strand exchange during homologous recombination. Rad 51 is the mammalian homologue to the bacterial Rec A protein and the yeast Rad51 protein(4). The high degree of conservation between prokaryotes and high order eukaryotes suggests the importance of this pathway for the cell. The homologos recombianation pathway includes a large number of proteins including RAD51, RPA, RAD52, RAD54, BRCA1,

BRCA2, XRCC2 and XRCC3(5, 6). Following recognition of the DNA damage and unwinding of the open ends of DNA, RAD 51 along with the RPA, RAD52 and RAD54 promote ATP-dependant DNA pairing and strand exchange (7-10).

Mice lacking BRCA2 and Rad51 have a Rad51-associated hypersensitivity to gamma radiation (11). BRCA2 deficient breast epithelial cells can survive with an unstable genome and thus proliferate, especially if they have another genomic alteration associated with malignancy. It is possible that loss of function of BRCA2 might increase genomic instability due to increased or error-prone homologous recombination mediated by Rad51. In this context, inhibition of Rad51 function may decrease the proliferative capacity of these cells. BRCA1 and BRCA2 mutations are estimated to be responsible in 80% of inherited cases of breast cancer and more than 95% of inherited ovarian cancers. While normal cells regulate the rates and activities of the homologous recombination (HR) machinery in check through BRCA2, tumor cells that are BRCA null will have a deregulated HR pathway. Thus, inhibition of homologous recombination in these cells will preferentially sensitize them to treatment. Being is the limiting factor for homologous recombination in mammalian cells(12), RAD51 is a good target for inhibition.

The reverse yeast two hybrid system

To identify inhibitors of the RAD51 protein we decided to use a genomic approach, and employed a modification of the yeast two hybrid system. Since protein-protein interactions are critical to almost every cellular process from cellular macrostructures to enzyme complexes and signal transduction, disruption of these interactions will provide a mechanism of deregulation of the respective pathways and hence a molecular target for drugs. Short peptides of a few amino acids (5-10) have been shown to be sufficient to destabilize protein-protein interactions. Thus a library of random combinatorial peptides of sufficient complexity will in theory have an inhibitory molecule for any protein-protein interaction. We proposed to use the reverse two-hybrid system to isolate inhibitory peptides of RAD51 self association (13). The system selects for a protein/peptide that will destabilize a protein protein interaction. This is done by introducing a Ura3 gene under the Gal 4 promoter as one of the reporter genes. Basal expression of Ura3 is inhibited by engineering an Upstream Repressing Sequence (URS1) of Spo13 upstream of Ura3. Ura3 encodes Orotidine 5'phosphate decarboxylase, used in the biosynthesis of Uracil. Ura3 can also catalyze the conversion of 5 Fluoro orotic acid (5'FOA) into a toxic

product, 5-fluorouracil (5'FU). Accordingly, if we transform this host strain with plasmids harboring genes whose products are known to interact (as fusions with the Gal 4 DNA binding domain and Gal 4-activation domain) the yeast will be able to grow in a media lacking uracil. However, the strain will be sensitive to the presence of FOA. Thus, if we now introduce a library of random peptides one of which may potentially inhibit this interaction in this cell, we may suppress the expression of Ura 3. A cell harboring such a peptide will be Ura^{-} FOA^{R} and thus can be selected.

The yeast strain we used was MaV103 (MATalpha leu2-3, 112 trp1--901 his3D200 ade2-101 gal4D gal80D SPAL10::URA3 GAL1::lac Z Gal1::his3@LYS2 can1R cyh2 R) (13). The host strain has 3 stably integrated Gal4p-inducible genes: SPAL::URA3, integrated at URA3, Gal1::HIS3, integrated at LYS2, and GAL1::LacZ integrated at an unknown locus.

HYPOTHESIS

Deficiency in DNA repair will sensitize cells to DNA damaging drugs and as such inhibitors of the DNA repair pathway could be potentially used as sensitizing agents in combination with traditional DNA damaging agents such as topoisomerase inhibitors and ionizing radiation.

Protein-protein interactions are critical in every cellular process and small peptides 10-15 a.a in length can potentially break these associations between proteins. Peptides that target specific interactions between critical proteins in the DNA repair pathway can then be used as inhibitors of the DNA damage repair. Such peptides can be identified by screening a random plasmid library coding for 15 a.a peptides in a modified reverse two hybrid screen.

OBJECTIVES

In order to identify inhibitors of the DNA repair pathway we wanted to use the reverse two hybrid system. The specific aims of this part were:

1. To develop yeast clones containing plasmids expressing Rad51 as a fusion protein with Gal4 activation domain (RAD51 AD) and Gal4 DNA binding Domain (RAD51 DB). The clones expressing RAD51 fusion proteins would be tested to determine the strength of Rad51 association in a two-hybrid context using Lac Z assays. The minimum concentration of FOA

required for cell killing of the Rad51 fusions transformed MV103 strain would then be determined.

2. To construct a plasmid library coding for random 15 amino acid peptides by cloning d.s DNA amplified from a pool of 77 nt synthetic oligos containing a 45 nt random region into the vector pHANLS.

3. To transform the library from step 3 into the yeast clone (from step1) containing the RAD51-AD and RAD51-DB and select at the minimum FOA concentration. We would then select for clones harboring plasmids that code for inhibitory peptides of RAD51 self association. The plasmids would then be rescued and tested independently for inhibitory activity.

MATERIALS AND METHODS

Yeast strain and plasmids: The yeast strain MV103, plasmids pHANLS, pGDBMAD and Rad51 cDNA expression vector were kind gifts from Dr. Zhiyuan Shen, Department of Molecular Genetics and Microbiology, Univ. of New Mexico.

PCR primers and random oligo synthesis : The 45 nt long random oligo was custom synthesized by Invitrogen (Carlsbad, CA). A 77nt long synthetic oligo containing a 45 nt random fragment was custom synthesized by IDTDNA technologies (Coralville, IA). The random fragment was generated by adding dNTPs in a ratio adjusted to their incorporation efficiency in chemical synthesis. Every third nucleotide incorporated in the random fragment was dGTP or dTTP inorder to accommodate the third base redundancy in codon usage.

Lac Z Assays: Colonies of transformed yeast strains were replica plated onto filters and grown at 30° C. After growth, the filters were removed and frozen in liquid nitrogen for about 20 seconds. The filters were thawed and incubated in 400 µl/plate of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) with XGal (bromo-chloro-indolyl-β-D-galacto-pyranoside). Each filter was incubated on a wet blotter until blue color development seemed sufficient, at which point the time was noted and the filter was removed and allowed to dry.

Transformations: Library transformations were carried out using the electroporation method and commercially available electroporation competent *E coli* DH10B cells (Invitrogen, Carlsbad CA) according to manufacturer's recommendation. Yeast transformaton were carried out using the Lithium acetate method (14, 15) following a protocol from BD biosciences.

RESULTS

The Reverse Two hybrid Set up: Rad 51 fusions were constructed with the Gal 4 activation domain (Gal4 AD) and the Gal4 DNA binding domain (Gal4 BD). The insert was PCR amplified and cloned into the pGDBMAD vector. Three plasmids were constructed: pGDBMAD/AD51 (Gal4AD-Rad51 fusion), pGDBMAD/DB51 (Gal4DB-Rad51 fusion), pGDBMAD/MAD51- (Gal4AD-Rad51 & Gal4DB-Rad51 fusion). The vectors were then transformed into MV103 and the transformants selected on Trp$^-$ plates. The clones obtained were then assayed for Lac Z activity (Fig. 1 a).

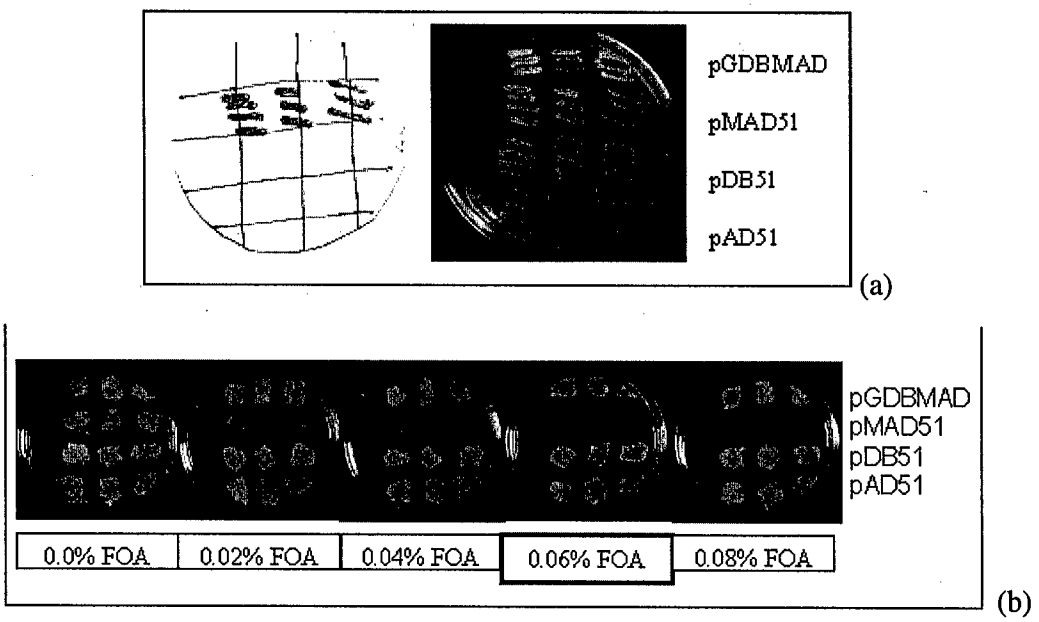


Fig 1 (a). Lac Z assay. 3 clones from each transformation (pGDBMAD, pMAD51, pDB51 and pAD51) were grown overnight. A colony lift Lac Z assay was then performed and color development was observed after 3 h. Only clones that harbored pMAD51 tested positive. **(b) Determination of minimum FOA concentration.** Yeast clones harboring different Rad51 fusion plasmids were plated in different concentrations of FOA. The minimum concentration of FOA to completely kill cells harboring both the DNA binding and activation domain fusion of Rad51 (pMAD51) was determined.

Determination of FOA concentration: Three clones from each transformation were taken and grown on Trp$^-$ plates containing 0%, 0.05%, 0.1%, 0.16% and 0.3% 5FOA by weight. Clones harboring pMAD51 are sensitive to 5' FOA and are killed between 0.05% and 0.1% FOA. In the next step the clones were plated on plates containing 0%, 0.02%, 0.04%, 0.06% & 0.08% FOA. Minimum FOA concentration for cell death in MV103/pMAD51 was 0.06% (Fig. 1 b). This concentration was determined to be the optimal concentration for selection of inhibitory peptides after transformation of the random library.

Construction of a combinatorial Library: We needed to construct a random DNA library coding for all possible combinations of 15 amino-acid peptides. We used the vector pHANLS, derived from the commercially available pGAD vector. The Gal4 activation domain was removed and a nuclear localization signal (NLS) was added upstream of the multiple cloning site (MCS) at the *Nco I* restriction site. A HA tag was added immediately downstream of the NLS. We tried three different approaches to construct the random library (Fig. 2).

Approach 1: A template for the library (RanTMP) containing N_{45} flanked by 15 bases on either side for primer binding and restriction was custom synthesized from Invitrogen (Carlsbad, CA). The library was then amplified by PCR using Ran TMP as template, precipitated using ethanol, digested at the introduced restriction sites and cloned into pHANLS vector. However this approach did not work well and the tranformation efficiency was always low (the maximum complexity obtained was in the order of 10^3), possibly due to selective amplification from the random template.

Approach 2: We then decided to use Klenow fill-in to generate our library. We used the same template as before but used Klenow enzyme and a single primer at the 3' end. The product was then purified as before and the ligation and cloning was carried out. However, we encountered new problems at this step. The small insert size (47 bases after restriction) provided a lot of background with no or multiple inserts. So we chose approach 3.

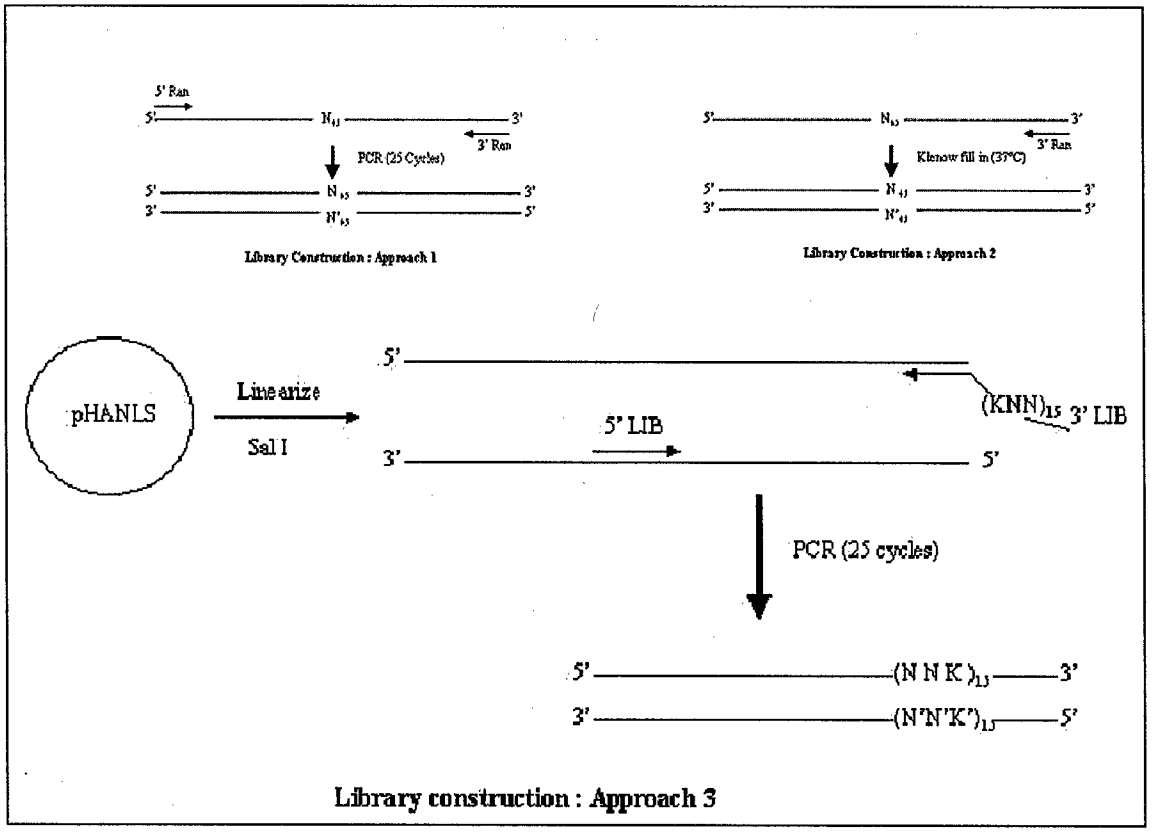


Fig 2. Approaches for Library construction.

Approach 3: We used a 77 nucleotide DNA fragment as the template for the library. The 3' end of the oligonucleotide is complementary to the vector pHANLS and has 15 repeats of NNK ('N' stands for any nucleotide and 'K' for the base 'A' or 'T'). Each 'NNK' serves as a codon for amino acid synthesis. Using this oligo as the 3' primer and a 5' primer from the upstream portion of pHANLS we generated a double stranded DNA fragment with the library at the 3' end. This approach addressed a variety of problems encountered in the previous approaches:

- Accomodating for the degeneracy of the codon usage allowed us to reduce the complexity required to cover the entire sample space by an order of 2^{15}.
- Incorporating the library as a primer prevented the amplification bias introduced by PCR.
- This method also allowed us to synthesize the library as a much larger fragment and

made it easier to handle and manipulate the library DNA during construction.

Using a restriction site incorporated in oligo primers we were able to directionally clone the library into the vector. Scaling up at this level gave us a library complexity of $5*10^6$ (Fig. 3).

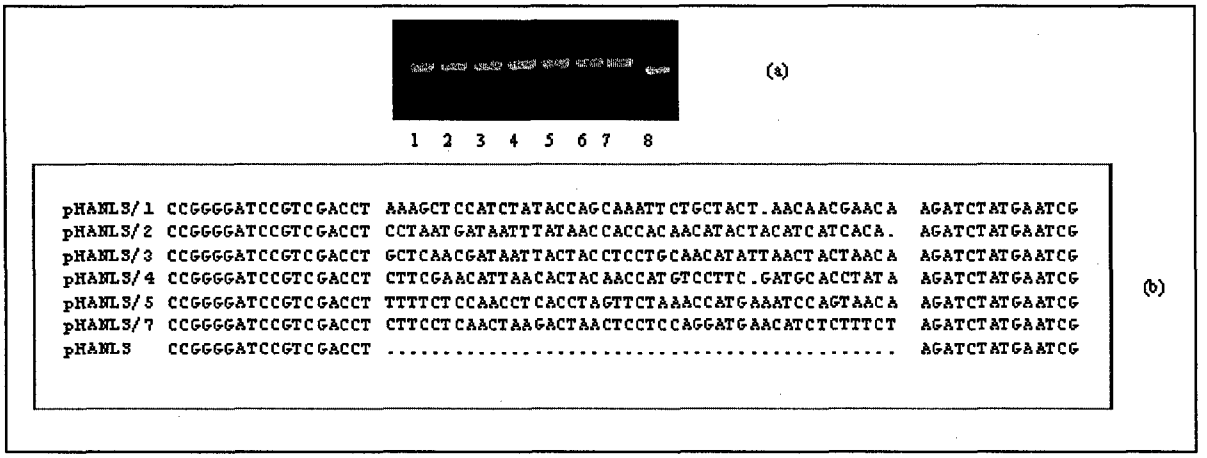


Fig 3. (a) PCR for random library inserts. Colony PCR was performed using primers in the vector pHANLS to analyze library inserts. A vector with no insert gave a band 48 bp smaller that (Lane 8) a plasmid harboring a random fragment. (Lanes 1-7). **(b) Randomness of library shown by sequencing.** 45 bp random insert flanked by vector sequences. Last sequence is empty vector.

We then transformed the library into the strains developed (MV103/pMAD51) described before. We had distinct problems at this stage that we were unable to overcome. The transformation efficiency in yeast is very low. We were not able to obtain efficiency of more that 10^5. This meant that we could never screen the whole range of the possible peptides encoded by our library. The number of clones that we were able to isolate were also too few in number. When the plasmids from these were rescued and reintroduced in the parent strain of yeast, there was no inhibition of Ura3 activity. Since none of these strains survived 5'FOA selection when the rescued plasmids were reintroduced, these were determined to be false positives. Accordingly we decided to terminate this part of the project because of these technical difficulties that we could not overcome (A statement to this effect was made in our annual report Y2 in which we also listed the modified objectives). We refocused our efforts on an in-silico approach to screen for potential inhibtors of the Ku70/80 protein, an important component of the NHEJ pathway and is detailed in the following chapter.

IDENTIFICATION OF NOVEL INHIBITORS KU70/80 DNA REPAIR COMPLEX AS POTENTIAL DRUGS IN CANCER TREATMENT

As stated previously there are two major mechanisms of repairing double strand breaks- the homologous recombination pathway and the non homologous end joining pathway (NHEJ)(16). The primary sensor of the NHEJ pathway is the trimeric complex of Ku70, Ku80 and the DNA dependant protein kinase, DNA-PK(3, 16). Being the primary sensor of this pathway, abolition of Ku activity severely impairs DNA repair ability by NHEJ (2, 17). The Ku knockout mice have a SCID phenotype and are also sensitive to DNA damage(18, 19). A dominant negative mutant of Ku70 that can dimerize with Ku80 but is deficient in DNA binding has been shown to sensitize HeLa and MCF7 cells to drugs that cause DSB(2). Also cells null for Ku70 or Ku80 show a 25 fold sensitization to bleomycin, a 10 fold sensitization to adriamycin and a 4 fold sensitization to VP16(20). Furthermore, because of their inability to correctly rejoin V(D)J recombination intermediates, animals lacking Ku or DNA-PKs display severe-combined immunodeficiency (21). A protein kinase cascade connects the detection of DNA damage to the activation of transcription factors, which in turn regulate the expression of genes involved in DNA repair leading to either cell cycle arrest and programmed cell death (apoptosis). The ataxia telangiectasia, mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) play major roles in the sensing of DNA damage and initiating subsequent cell signaling (22, 23). The decision by the cell to undergo growth arrest or apoptosis is the result of complex signaling events and is based on the extent of DNA damage and its repair. Blocking DNA repair will potentially induce the cells to undergo apoptosis instead of growth arrest. The DNA repair pathway is thus an obvious target for a sensitizing agent to DNA damage inducing drugs. Deficiency in DNA damage repair will sensitize cells to DNA damaging agents and thus such tumors can potentially be treated with a lower dose of chemotherapeutic agents/radiation. We wanted to use the structure of the Ku70/80 dimer to screen for small molecule inhibitors with high potential for binding the DNA binding pocket of this protein complex. The Ku70/80 complex is the critical first step in the NHEJ pathway. The DSB is recognized and bound by Ku70/80 which then assembles the DNA repair complex on the open ends of the DNA. Ku70/80 is also the regulatory subunit of the DNA dependant protein kinase DNA-Kc, which is responsible for transducing signalling mechanisms following DNA damage. Thus Ku70/80

inhibition would potentially abolish the NHEJ pathway, sensitizing the cells to DSBs.

Structure of the Ku70/80 Complex

The crystal structure of the Ku70/80 in complex with DNA has been published (24). The structure clearly delineates important motifs of the Ku proteins that are responsible for DNA binding activity. The Ku70/80 complex has a preformed ring structure that is of the same diameter as a d.s DNA molecule.

By forming a ring around DNA, Ku achieves high-affinity binding to open-ended double strand breaks while preventing the complex from binding randomly to closed DNA. Also there is a polarization of positive electrostatic charge on the inner surface of the ring and along the DNA-binding cradle. These positive-charged amino acids are critical for interactions with the phosphate backbone of DNA. Comparison of the DNA-free and DNA-bound crystal structures shows that the ring maintains its conformation in the absence of DNA. The crystal structure makes clear that Ku binds the DNA in a sequence-independent manner through a limited set of interactions with the sugar-phosphate backbone without a single interaction with a nucleotide base. This sequence independence in binding DNA is also essential for the Ku Complex to nonspecifically bind d.s breaks.

Virtual Ligand Screening using DOCK

With the availability of fast computers and commercially available structure databases of small compounds, virtual screening technologies using high throughput docking have become very economical. Docking is the ability to position a ligand in the active or a designated site of a protein and calculate specific binding affinities. Docking approaches offer several fold enrichment in finding a novel investigative lead over conventional screening techniques such as high throughput screening (HTS) (25, 26). We used the freely available DOCK 4.0 (http://dock.compbio.ucsf.edu/dock4/dock4.htm) suite of programs for our virtual ligand screening. The DOCK software consists of four main programs – *ms, sphgen, grid* and *dock.* The first step is to generate a molecular surface for the active site. The dot surface is generated using *molecular surface* (*ms*) program. Next the *Sphgen* provides a negative characterization of the target site. The shape of cavities in the receptor is used to define spheres; the centers of the spheres become potential locations for ligand atoms. This sphere clusters generated are then viewed graphically to identify the cluster which is closest to the target site on the protein used for

docking. The program *grid* creates the grid files necessary for rapid score evaluation in the *dock* step. The *grid* program generates information about the steric and electrostatic environment surrounding the target site of interest. The final step is *dock*, which evaluates each individual compound from the database of small molecules for fit into the target site. Three types of scoring are provided in the DOCK program: contact, chemical and energy scoring. A flowchart outlining the steps involved is given in Fig 4.

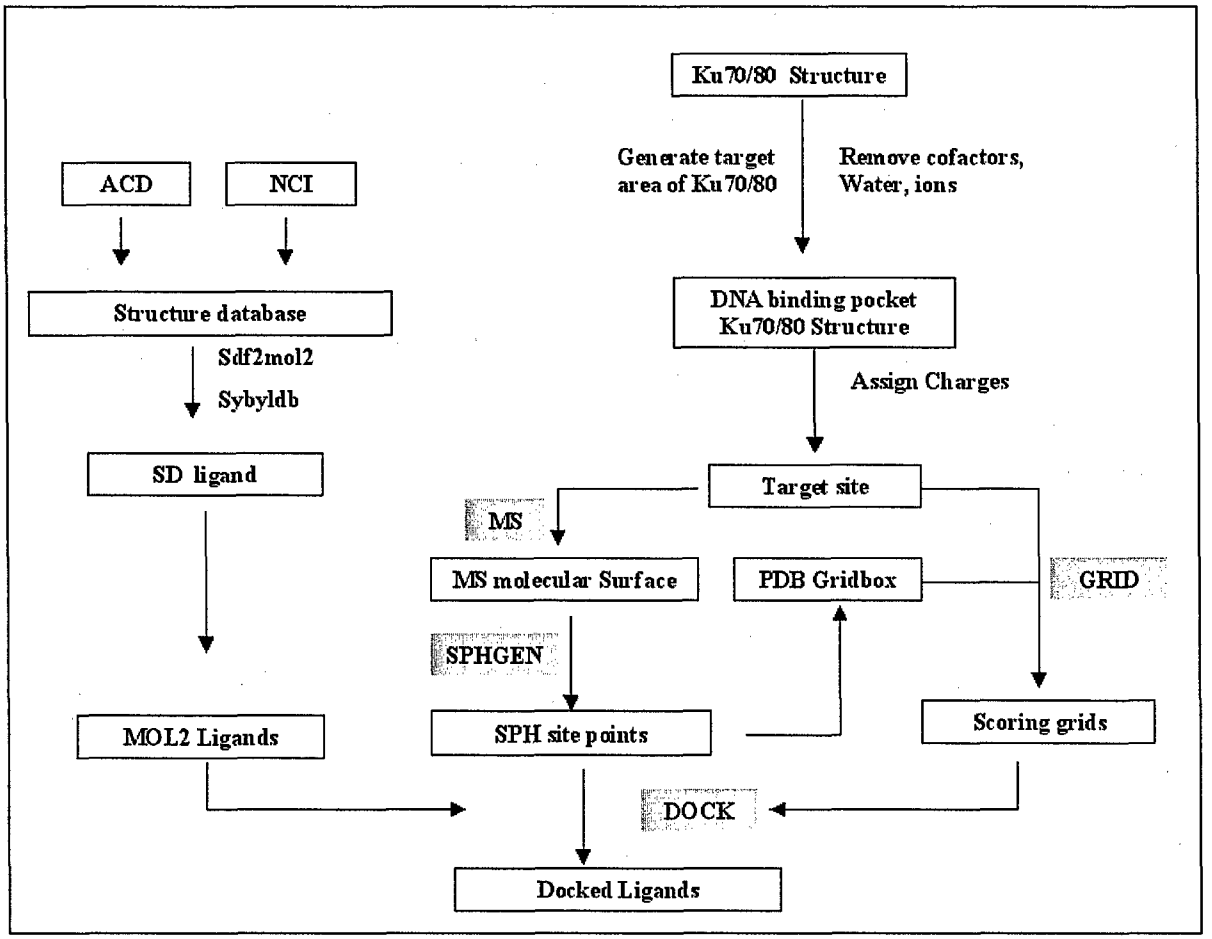


Fig 4. Flowchart for Virtual Ligand Screening using DOCK.

HYPOTHESIS

The Ku70/80 dimeric protein is the critical first step in the non-homologous end joining pathway (NHEJ) of DNA repair. Inhibition of Ku70/80 DNA binding will aboloish DNA repair through NHEJ. It is possible to screen for potential inhibitors of the Ku70/80 DNA binding activity by identifying the region important for DNA binding from the Ku70/80 crystallographic structure and performing a virtual ligand screen from a database of small molecule 3D structures on the DNA binding pocket of Ku70/80. Molecules having the highest shape complementarity and electrostatic attraction potential to the target area on the Ku70/80 comlpex would have a high potential to bind and inhibit its DNA binding activity. The top scoring compounds identified by this method can then be quickly evaluated for biochemical activity in-vitro.

OBJECTIVES

The goal of this part of the project was to rapidly screen and evaluate potential inhibitors of the Ku70/80 DNA repair protein complex using virtual ligand screening of 3D structure databases and the DOCK software. The speific aims were:

1. To characterize and identify a critical pocket on DNA binding region of the Ku70/80 complex from available structural information
2. To target this site for virtual ligand screening using a database of small molecule 3D structures and the software DOCK.
3. To test the highest ranked small molecules from the DOCK screen based on the energy score (electrostatic attraction) for biochemical activity.
4. To evaluate positive hits from the biochemical screen in cell culture for sensitizing tumor cells to DNA damaging agents.

MATERIALS AND METHODS

Protein Structures: The coordinates for the crystal structures of the Ku70/80 dimeric complex in the presence (1JEY) and absence of DNA (1JEQ) were obtained from the Protein Databank (PDB, http://www.rcsb.org/pdb/).

Virtual Ligand Screening: Virtual ligand screening was done using programs from the DOCK software. The site targeted for virtual ligand screening was defined by the DNA binding region of Ku70/80 with maximal interactions with the DNA backbone. Ligand docking was carried out as recommended by the licensor. Two databases of molecule structures were used: Available Chemicals Database (ACD from Molecular Design Ltd., San Leandro, CA) and the NCI database. 2D coordinates (downloaded from http://cactus.nci.nih.gov/ zncidb2/download.html) were converted into 3D structures using CONCORD™ (Tripos Inc., St. Louis, MO). Programs from the DOCK suite (27) were used to define the target site and score ligands for electrostatic and contact affinity. The top 500 electrostatic scorers were clustered according to structural diversity using the Sybyl Selector™ package (Tripos Inc., St. Louis, MO).

Assay for testing inhibitors of Ku70/80: The compounds to be tested were dissolved in DMSO or PBS and tested using 3 different concentrations (75, 150 and 225 μM). Briefly 10 pM of biotinylated ds DNA was immobilized on streptavidin agarose beads (Pierce Biochemicals, Rockford, IL). The beads were then incubated with 100 μg of total protein extract in the presence of the test compound or diluent for 1h. Proteins pulled down with the immobilized DNA were then washed in protein extraction buffer (100 mM Tris pH 8.0, 100 mM NaCl, 10% Glycerol, 1mM EDTA, 0.5% NP40, protease inhibitors), separated by polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a nitrocellulose membrane and western blotting was performed using a monoclonal Ku80 antibody (BD biosciences, San Jose, CA).

Cell Viability assays: The drug sensitivity of cells was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay), described previously (28). Briefly, PA1 (human ovarian cancer cell-line) cells were seeded at 2500 cells/well in 100 μl of culture medium in 96-well plates. The following day, cells were then treated with serial dilutions of VP16 (0, 0.4, 2 and 10 μM) and test inhibitor of Ku70/80 (0, 1, 5 and 25 μM) in triplicate. 72 h later, the media containing the drugs was removed from each well, and the MTT reagent (25 μl/well, 2 mg/ml; Sigma) was added. After incubation at 37°C for 3 h, the medium was aspirated, and 100 μl of DMSO was added to solubilize the formazan. Optical density at 560 nm was measured using a microplate reader (Molecular Devices, Sunnyvale CA). Mean values (± SD) of two independent experiments were plotted using GraphPad Prism package (GraphPad, San Diego, CA).

RESULTS

Virtual Ligand Screening: The topology of the Ku70/80 ring structure shows the polypeptides involved (Ku70, residues 277-341 and Ku80, residues 267-336). The amino acids maintaining the DNA backbone interaction are R80, S78, A255, S257, R258, N275, Q278, K338, R403 and R404 for Ku70 and R242, K265, T275, W276, Y397, D398, R400, A401, N402, R431 and R486 for Ku80. The region bounded by these amino acids provides an ideal target site for an inhibitor of Ku70/80 DNA binding. This pocket, designated pocket 157 is illustrated in Fig 5. The pocket 157 was used as the active site for the DOCK process. Using the pocket 157 as the target site of interest, virtual ligand screening was carried out using programs from DOCK. We performed three screens overall. For an initial screen, the ACD database was pre-screened for compounds with drug like properties based on molecular weight, solubility and charge density. In a second screen all ACD compounds that were above 100 daltons in molecular weight were used for the DOCK screen. The third screen was performed using the NCI database. Ligands were scored based on electrostatic binding affinity (energy score) and shape complementarity (contact score). Each ligand was scored for a maximum of 50 orientations. The top 500 energy scorers were further analyzed. Among the top scorers were a variety of nucleoside analogs (UTP, dATP) that served as built-in positive controls and provided validation for the scoring functions as well as the target site on the Ku complex. The top 500 scorers (based on Electrostatic Score) from each of these screens were then clustered according to structural diversity using the Sybly Selector™ package. Forty compounds (20 from ACD and 20 from NCI3D) were selected so as to represent different structural classes to test for biochemical activity. The compounds that were selected for testing in for biochemical activity are listed in Table I and II.

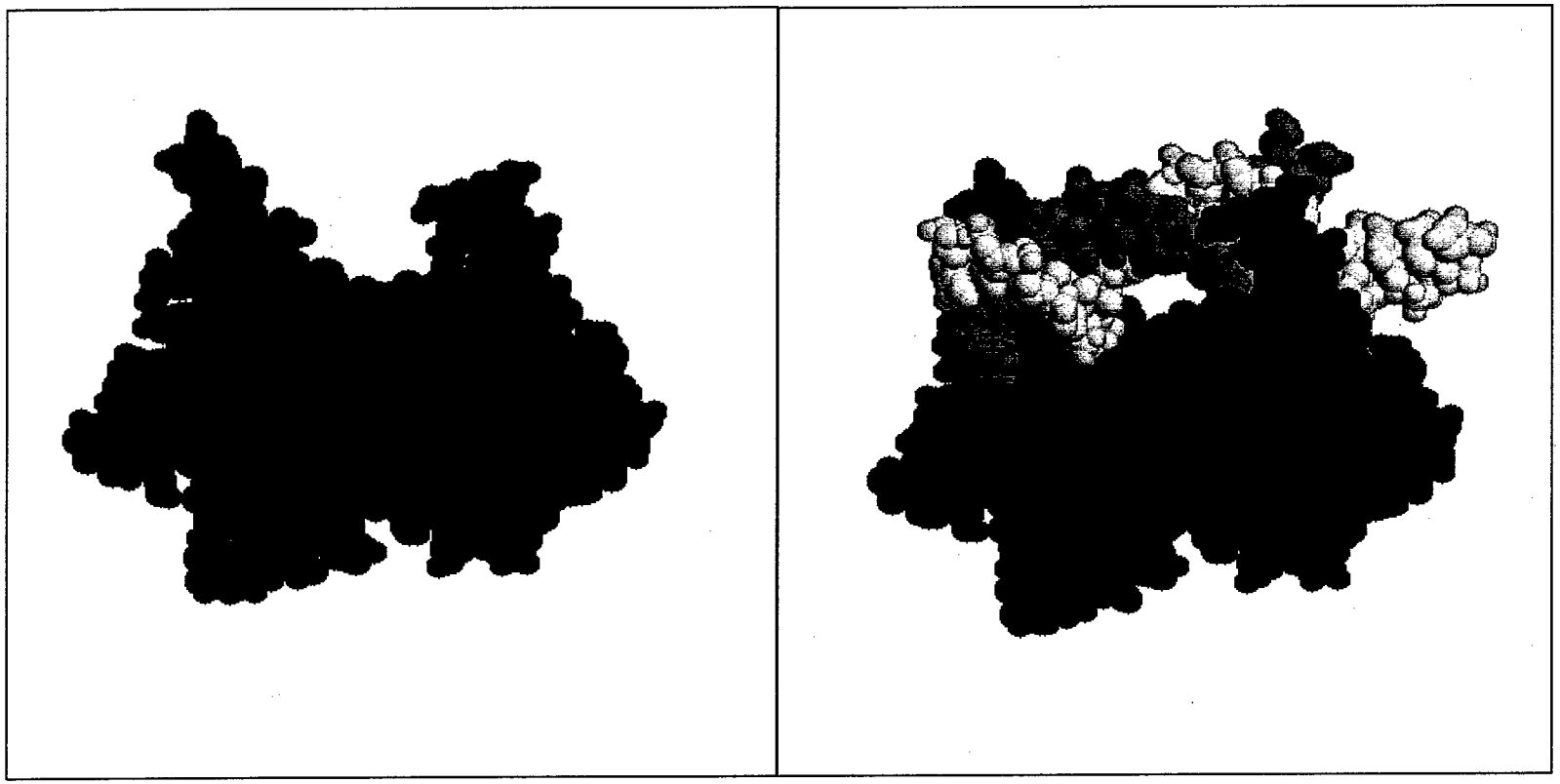
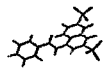
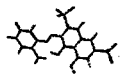
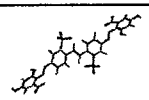


Fig 5. Target site for Virtual Ligand Screening. Ku70/80 DNA binding pocket is shown. Pocket without DNA is on the left and pocket bound to DNA is on the right. Ku70 is in red and Ku80 is in blue, DNA is in green and yellow. The structure file has been edited to show the target site.

NCI COMPOUNDS CHOSEN FOR TESTING FOR BIOCHEMICAL ACTIVITY

NCI No	Structure	Score	Rank
84137		-131.5	3
42164		-93.05	41
100234		-89.99	62
3766		-86.41	94
37172		-80.56	156
89166		-80.41	162
634748		-79.19	187
13977		-79.01	193
45195		-78.14	212
377645		-77.94	217

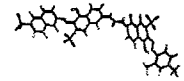
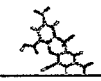
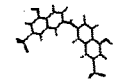
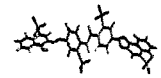
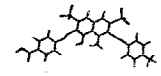
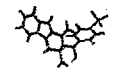
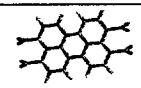
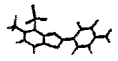
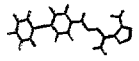
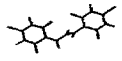
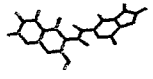
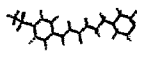
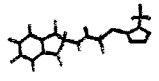
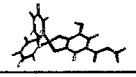
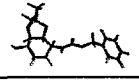
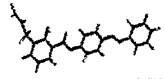
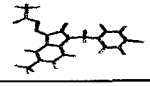
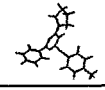
NCI No	Structure	Score	Rank
65575		-77.11	244
87216		-77.03	247
43325		-75.64	277
37203		-74.24	341
37052		-72.71	409
34931		-72.52	415
45598		-72.37	424
623549		-72.24	430
629709		-71.71	444
89768		-71.63	463

Table I. NCI compounds tested. Structures of NCI compounds tested for biochemical activity with their rank and scores from DOCK.

ACD COMPOUNDS CHOSEN FOR TESTING FOR BIOCHEMICAL ACTIVITY

ACD no	Structure	Score	Rank
MFCD00134235		-45.42	1
MFCD00182873		-33.76	7
MFCD00224373		-32.01	13
MFCD01570991		-32.54	10
MFCD02178839		-32.01	14
MFCD00203132		-31.91	15
MFCD01764045		-31.21	21
MFCD00183275		-29.01	87
MFCD00271311		-28.67	110
MFCD01863553		-28.46	124

ACD no	Structure	Score	Rank
MFCD00187061		-28.42	130
MFCD00141752		-28.09	157
MFCD01444203		-26.53	188
MFCD01567838		-24.14	209
MFCD00218607		-23.76	210

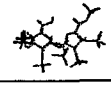
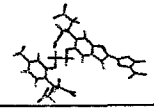
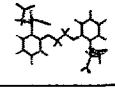
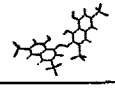
ACD no	Structure	Score	Rank
MFCD00270078		-65.43	24
MFCD0036956		-60.88	36
MFCD00183884		-54.2	72
MFCD00070080		-53.97	75
MFCD00046409		-53.13	86

Table II. ACD compounds tested. Structures of ACD compounds tested for biochemical activity. Two DOCK screenings were carried out, one for a smaller database of drug like molecules and another with all ACD molecules above 100 Daltons.

Biochemical Activity of DOCK hits: To test the high scoring hits from the DOCK screening for inhibition of Ku70/80 activity we developed a quick assay. Briefly we used streptavidin beads to immobilize 5' biotinylated double stranded (d.s) DNA and used it to pull down Ku70/80 complex from MCF7 total cell extract. The pulled-down proteins were then separated on a 7.5% polyacrylamide gel. The separated proteins were transferred onto a nitrocellulose membrane. The Ku70 or Ku80 can then be detected by western blotting. However, if any test compound inhibits this activity, the complex is not pulled down. We then showed that this assay is semi quantitative. Increasing immobilized DNA on streptavidin beads could increase the amount of Ku complex pulled down and increasing the amount of protein extract in the pull down also increased the amount of Ku complex pulled down. Importantly free double stranded DNA when present could compete out Ku complex binding to the immobilized DNA in a dose dependant manner. Thus this assay can be used to quickly screen a large number of compounds for biochemical activity of the Ku protein complex (Fig. 6). It should be noted that the assay conditions require a large amount of protein and DNA. This is because of the low efficiency of Ku70/80 pull-down by ds DNA and the low reslution of detecting proteins in a western blotting. The concentration of compounds to be tested should be appropriately high.

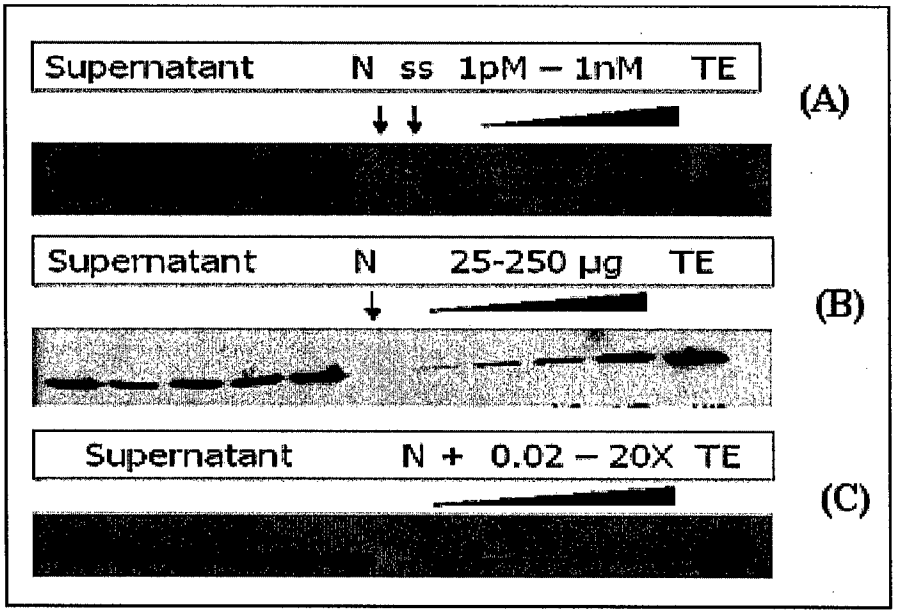
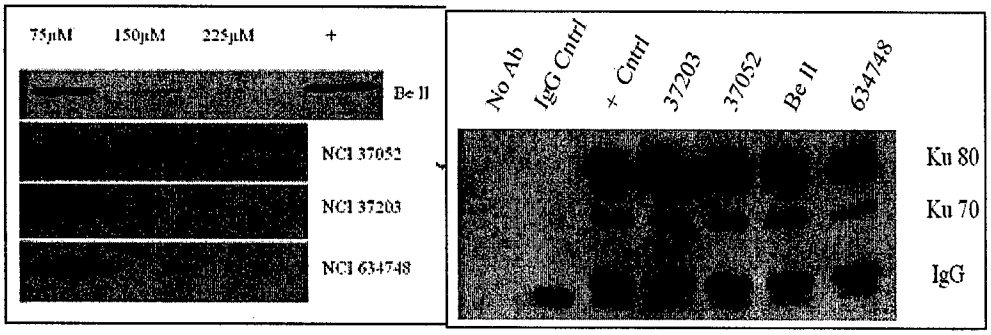


Fig 6. Bioassay for testing Ku70/80 DNA binding inhibitors (A) Increase in amount of double stranded DNA increases Ku complex pulled down, (B) Increase in Total protein used increases amt if Ku complex pulled down, (C) Amount of Ku complex pulled down can be competed out by free double stranded DNA in solution (N – Negative control, TE – Total Extract, ss - single stranded DNA)

We used this assay to screen for putative inhibitors of Ku70/80 DNA binding activity. As seen in Fig 7 (a), four compounds out of 40 tested had biochemical activity. Briefly, we used 10 pM of d.s DNA immobilized on agarose beads and 100 μg of MCF7 total protein extract. Each compund was tested at at least three concentrations (25, 150 and 225 μM). The concentrations at which the compounds were tested was high, but comparable to what is used in HTS(29). It reflects the sensitivity of the assay because a large amount of the Ku70/80 is needed in the protein extract inorder to be able to visualize the pull downs in a western blot. This does not reflect the concentrations at which these compounds need to be tested in cell line or other biological systems. One ACD compound (Berryllon II) and 3 NCI compounds (NCI 37052, NCI37203 & NCI 634748) inhibited Ku activity



(a) **(b)**

Fig 7. (a) Compounds having biochemical activity and their structures. 100μg of total protein extract and 10 pm of biotinylated DNA were used for the assay. Compounds were dissolved in DMSO and tested at atleast 3 different concentrations (75, 150 and 225μM). **(b) Structures of active compounds** (Clockwise from top left – NCI 634748, NCI 37052, NCI37203, Berryllon II). **(b) Inhibitors of Ku activity do not destroy protein-protein interactions**. Immuno precipitation reactions were carried out in the presence of 300μM of test compounds (Berryllon II, NCI 37203, 37052 & 634748) or diluent (DMSO) using mouse monoclonal antibody against Ku80. The precipitated proteins were then separated using PAGE. Westerm blotting was caried out using anti Ku70 and anti Ku80 antiboides. IgG heavy chain served as a loading control.

Specificity of Ku70/80 inhibitors: We needed to test the specificity of the inhibition of the Ku activity by these compounds. Since the inhibitors were all charged molecules, there was a possibility that the observed inhibition was a non specific effect mediated by the general disruption of all protein-protein interactions. To test this, we carried out immuno-precipitation reactions using antibodies against Ku80, separated the precipitates by SDS-PAGE, transferred

onto nitrocellulose and then probed for Ku70 as well as Ku80. The IgG bands served as a loading control. As seen in Fig 9 (b), there was no difference between the control reactions and the immuoprecipitations carried out in the presence of 300μM of test compounds. This proved that the inhibition was not due to the disruption of antibody-protein or other protein-protein interactions or any other issues with protein folding.

Cellular Activty of the active inhibitors: We then needed to verify whether the biochemically active Ku inhibitors were active in cell based assays and sensitize cells to DNA damaging agents. To this end we used the topoisomerase II inhibitor, etoposide (VP16), which stabilizes the transient DNA-protein covalent linkage formed during topoisomerase action and blocks religation of DNA, causing extensive double stranded breaks in the DNA. The DNA damage results in the activation of the apoptosis cascade and cell death. We used the MTT assay to measure cell viability, treating the cells to different concentrations of VP16 in combination with each of the Ku70/80 inhibitors for 72h. 2 compounds, NCI 37052 and NCI 37203, substantially sensitized the cells to the DNA damage caused by VP16 while having little toxicity by themselves (Fig 8). However, the other two compounds, Berryllon II and NCI 634648, were toxic to the cells and had only an additive effect on the PA1 cells (Fig 9). The IC_{50} values of VP16 at different concentrations of the Ku70/80 inhibitors are summarized in Table III.

As seen in Table IV, the NCI compounds, NCI 37052 and NCI 37203 showed a dose dependent increase in sensitizing PA1 cells to the DNA damaging agent VP16, while showing little toxicity by themselves. This sensitization is comparable to previously published results where immortalized Ku70 -/- and Ku80-/- mouse embryo fibroblasts (MEFs) showed a similar seistivity to DNA damaging agents including VP16 when compared to wt MEFs.

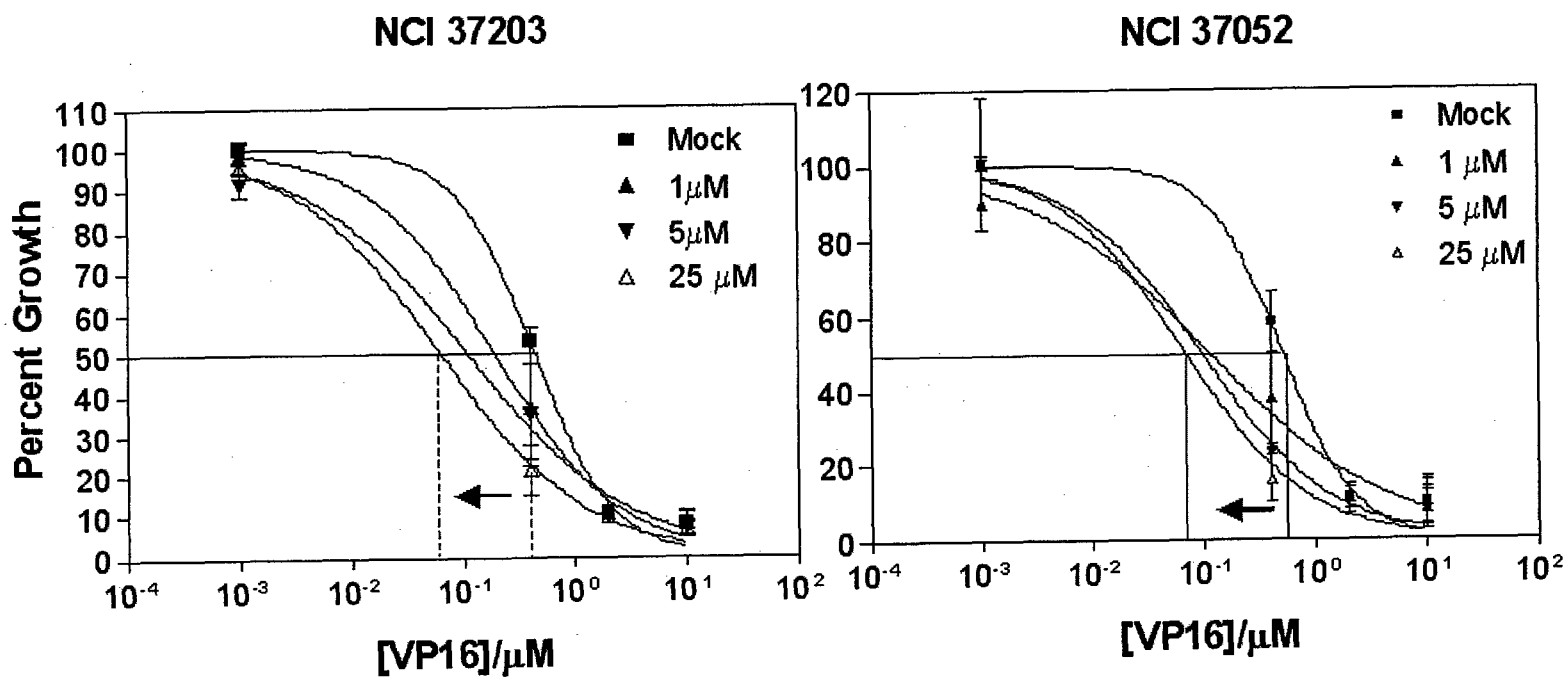


Fig 8. Cell viability assays for VP16 in the presence of Ku70/80 inhibitors. MTT assays were carried out for determining the contribution of the Ku70/80 inhibitors in sensitizing PA1 cells to the topoisomerase inhibitor VP16. 4 concentrations of the inhibitor were used (0, 1, 5 and 25 μM).

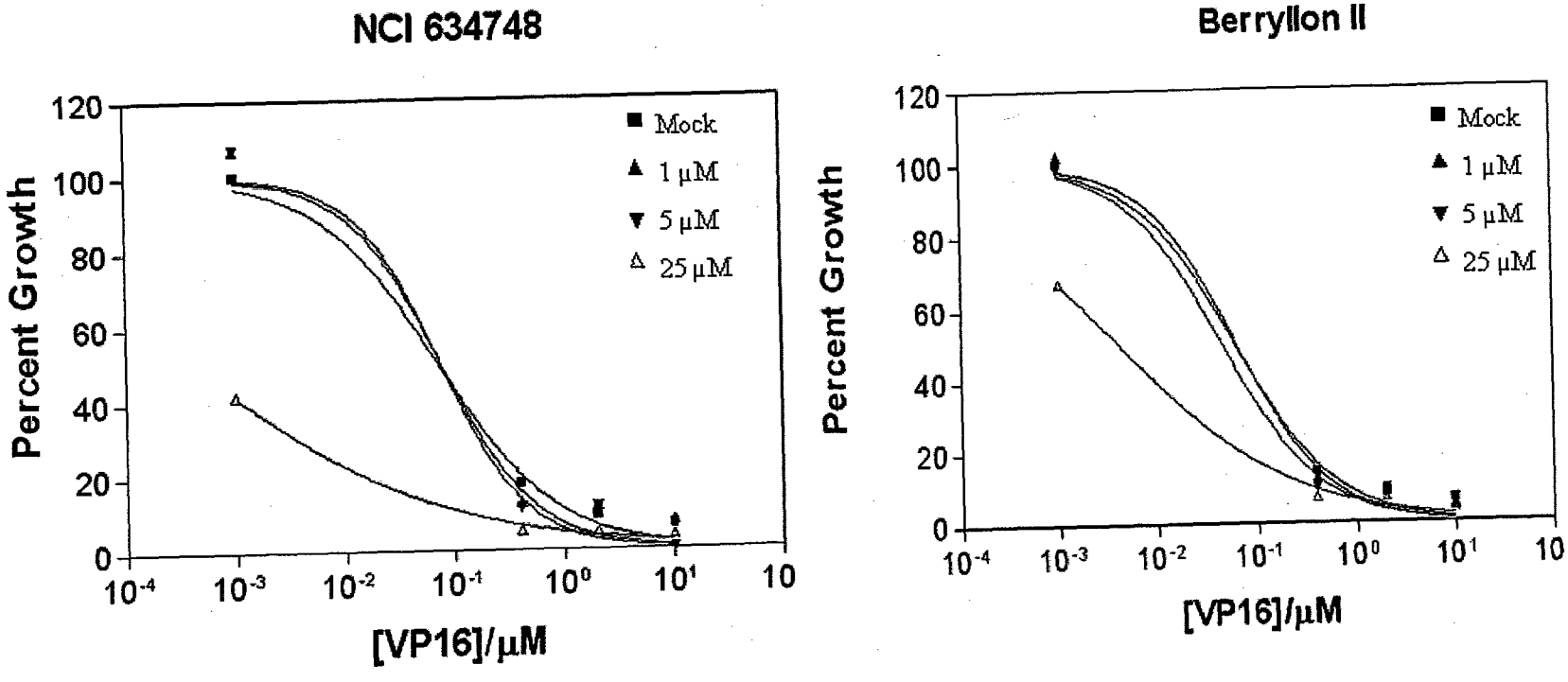


Fig 9. Cell viability assays for VP16 in the presence of Ku70/80 inhibitors. MTT assays were carried out for determining the contribution of the Ku70/80 inhibitors in sensitizing PA1 cells to the topoisomerase inhibitor VP16. 4 concentrations of the inhibitor were used (0, 1, 5 and 25 μM)

IC_{50} OF VP16 IN THE PRESENCE OF KU70/80 INHIBITORS

	0	1 μM	5 μM	25 μM
NCI 37052	0.502	0.110	0.094	0.068
NCI 37203	0.438	0.192	0.110	0.063
NCI 634748	0.072	0.077	0.0760	0.004
Berryllon II	0.061	0.0639	0.0460	0.004

Table III. IC 50 for VP16 in the presence of Ku70/80 inhibitors. MTT assays were carried out in the presence of 0, 1, 5 and 25 μM of each of the putative inhibitor of Ku activity inorder to determine their contribution to the IC_{50} for DNA damaging agent VP16. The IC_{50} for VP16 is listed for different concentrations of the Ku70/80 inhibitor.

DISCUSSION

In this study, we evaluated the usefulness of virtual ligand screening approaches to identify valuable lead compounds for the inhibition of the Ku70/80 DNA binding activity. Using the freely available DOCK software and NCI compound 3D structure library and the commercial ACD library of 3D structures, we were able to score compounds for potential inhibitory activity of the Ku70/80 complex DNA binding activity. High scoring nucleotide analogues acted as internal positive controls for the scoring functions used in DOCK. We also tested 40 of high scoring hits for inhibitory activity in a biochemical assay for Ku70/80 DNA binding activity. A high percentage (10%) of high scoring hits from DOCK also showed biochemical activity. Thus DOCK seems to offer several hundred-fold enrichment over conventional HTS in identifying potential inhibitors. We identified four compounds (three from the NCI structure database and one from the ACD database) as having inhibitory activity in a biochemical assay for Ku70/80 DNA binding activity. Two of these compounds also increased sensitivity to the DNA damaging agent VP16 while having little toxicity by themselves.

Our study shows the potential for using such a strategy to economically screen for inhibitors using virtual libraries and DOCK. Our study also identifies a novel target for cancer

chemotherapy i.e. the DNA repair pathway. We show that inhibition of the DNA repair mechanism in cancer cells will sensitize such cells to conventional DNA targeting drugs. These small molecules show promise as sensitizing agents for chemotherapy, allowing for a lower dosage of the conventional DNA damaging agents such as radiation or topoisomerase inhibitors. Damage to the cell's DNA will activate the stress response system, which includes the kinases ATM/ATR and DNA-PK and the activation of p53 and NFκB(30). The decision to undergo apoptosis or growth arrest depends on the extent of the DNA damage and the cell's ability to restore the integrity of the DNA. Thus, blocking the DNA repair machinery through the inhibition of the Ku70/80 DNA binding activity while allowing for the ATM/ATR kinases to be activated would decisively shift the damage response from inducing growth arrest to apoptosis. Identifying and evaluating inhibitors of important proteins of these pathways would thus be an important strategy to be evaluated for the successful treatment of cancer. Indeed KuDOS Inc. (http://www.kudospharma.co.uk, Cambridge, England) is using this very strategy by evaluating inhibitors of ATM, DNA-PK, PARP and mTOR, all components of the DNA damage response machinery, as both stand-alone agents and sensitizing agents for cancer chemotherapy.

Cancer cells are more sensitive to inhibition of DNA repair than normal cells for a number of reasons. Because they divide rapidly, they have less time to carry out DNA repair than normal cells; they often lack fully functional DNA repair mechanisms which increases reliance on those remaining; and finally, they usually replicate under conditions of extreme stress, increasing levels of endogenous DNA damage. Thus, cancer cells are very sensitive to the inhibition of DNA repair machinery. This provides high selectivity for DNA damaging drugs acting in combination with DNA repair inhibitors to kill only cancer cells while reducing the unwanted side effects that are normally associated with such agents.

A potential disadvantage of using the Ku70/80 inhibition as a means of sensitizing cells to the DNA damage pathway would be that inhibition of this activity would prevent activation of DNA PK which requires Ku70/80 DNA binding. But DNA PK is not the only transducer of DNA damage to the apoptotic machinery. Since damage-activated kinases play important roles in activating the apoptotic machinery, our novel method of sensitizing cells to DNA damage agents may not work effectively in cancers that have mutations in ATM/ATR. However, in cells that have non-functional homologous recombination pathway, inhibition of NHEJ will sensitize cells greatly to DNA damage. Thus, blocking DNA damage repair may not work effectively in

all cell lines or tumors. Further studies need to be done to identify under which conditions such inhibitors have the greatest efficacy.

Molecular docking as a screening tool has many practical advantages. Docking tools like the one we used here can reliably screen out compounds that do not fit in a binding site or that have grossly wrong electrostatic properties. This allows for careful experimental assays on a relatively small number of database compounds, especially in cases where there are no high throughput assays available. Finally, a docking hit comes with a prediction of geometry, and this allows for inhibitor optimization in the context of a binding site. Docking tools offer several hundred fold enrichment in identifying novel inhibitors when compared with HTS (29). This is also borne out by the fact that 10% of the compounds we tested showed biochemical activity while the average hit rate for HTS is less that 0.025%. Also our study is the first time docking and virtual ligand screening has been used to identify inhibitors of large multi subunit complexes like the Ku70/80 complex. Our study shows that DOCK can be used effectively and economically for such complexes, where difficulty in protein purification would make assays for HTS unfeasible. This study also highlights the strategy we have used to identify inhibitors of DNA repair as effective sensitizing agents for DNA damaging drugs.

Key research accomplishments

1. A random DNA library of complexity in the order of 106 encoding 15 amino-acid peptides was synthesized.
2. A system to select inhibitors of Rad51 self association in the context of a Reverse Two hybrid System was set up.
3. Critical interactions for Ku70/80 DNA bindng activity were mapped and DOCK screens were carried out to identify potential inhibitors of the Ku70/80 DNA binding activity.
4. An assay for testing the inhibitors of the Ku activity was established.
5. 40 compounds selected from the top hits from the DOCK screen were tested for biochemical activity in the assay developed inhouse and 4 novel inhibitors of the Ku70/80 activity were identified.
6. Two inhibitors of Ku70/80 sensitized PA1 cells to the topoisomerase inhibitor VP16.

Reportable Outcomes:

Poster: S. Kamalakaran, W. TaoFu, M. Johnson, & WT Beck. Identification of inhibitors of Ku70/80 activity by Virtual Ligand Screening, AAPS Pharm Sci., Vol.4, No.4, M1182, 2002.

Poster: Kamalakaran S & Beck WT, Identification of inhibitors for protein-protein interactions involved in DNA Repair as potential drugs in breast cancer, Dept. of Defense Era of Hope Breast Cancer Meeting, Orlando, FL, 2002.

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CONCLUSIONS

In this project, we have evaluated two strategies for identifying inhibitors of the DNA repair pathway. We used the yeast reverse two hybrid system to screen for inhibitory peptides of the Rad51 protein in order to disrupt the homologous recombination pathway from a plasmid library coding for 15 amino acid peptides. While we succeeded in generating a library of sufficient complexity, because of inherent technical issues involved we were not successful in identifying the inhibitory peptides. For identifying inhibitors of the Ku70/80 complex (the important component of the NHEJ pathway) we used a structure based virtual ligand screening approach. We identified four of the high scorers from the DOCK screen as having biochemical activity in a Ku70/80 DNA binding activity assay. Our study show that the percentage of highest scorers from the DOCK screen that test positive for inhibitory activity in biochemical assays is several-fold higher than in conventional high throughput screens (10% in our DOCK screens over <0.03% for high throughput screening). Also, two of the compounds we identified using this approach effectively sensitize cancer cells to DNA damaging agents while having little toxicity themselves.

Our results demonstrate the validity and importance of both our target as well as the approach we used to identify inhibitors of the target. Targeting the DNA repair pathway seems to be an effective way to sensitize cells to conventional DNA damaging agents such as topoisomerase inhibitors and ionizing radiation. Structure-based drug screening using virtual libraries may be a useful and economical way to identify inhibitors of large multi subunit complexes where establishing assays for HTS may be difficult.

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